PRESENT STATUS OF ATTENUATED LIVE VIRUS POLIOMYELITIS VACCINE*

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THE obvious reason for trying to learn all we can about the possibility of immunization with an oral, living attenuated poliovirus vaccine, is to see whether or not it may be possible to reproduce the long-lasting immunity conferred by natural infection without the varying associated risk of paralysis. There are also the secondary considerations of the advantages of a vaccine that can be given by mouth instead of by injection, and of the possibility that widespread oral use of highly attenuated polioviruses might eliminate the naturally occurring virulent strains just as the smallpox virus was eliminated from many parts of the world by the use of the living vaccine against smallpox.

Koprowski and his associates[1] were the first to show that feeding of a type 2 poliovirus of diminished intracerebral virulence for monkeys could produce an immunogenic alimentary infection in human beings. The excellent subsequent studies of Koprowski and his associates[2] with two type 2 strains and one type 1 strain, as well as my own studies[3] with a number of different strains of each of the three types of poliovirus have established beyond doubt that immunization of human beings by the oral route is not only possible, but also that in the several hundred humans used in both studies this occurred without any significant symptoms. Much has now been learned about the multiplication of various attenuated strains, about dosage, about interference when different types are fed simultaneously, about multiplication in the presence of passively or placentally transmitted antibody, or in the presence of antibody produced by formalinized vaccine, about propagation of such viruses on a large scale *in vitro* as well as about the changes which may occur during their propagation *in vivo*. The crucial question now is not whether oral immunization against poliomyelitis is possible but rather

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FACTORS IN QUANTITATION OF VIRULENCE OF POLIOMYELITIS VIRUSES BY INTRACEREBRAL AND SPINAL INOCULATIONS OF MONKEYS

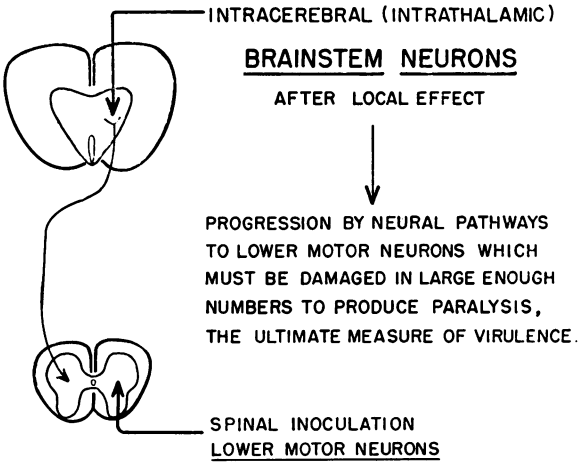

Fig. 1

what kind of attenuated strains may justifiably be used on increasingly larger numbers of human beings in the stepwise tests which must precede any trial of such a vaccine on a large scale. The important question here is obviously what constitutes attenuation, how to measure it and how to control it. During the past four years this has been the chief problem under investigation in my laboratory and approximately 9,000 monkeys, 150 chimpanzees and 133 human volunteers have been used thus far in the quantitative studies of the various characteristics of different strains of polioviruses.

In nature paralytic infection among humans has varied from a low of about 1 per 100,000 per annum to a rare peak of about 20 per cent which was observed among an isolated, highly inbred group of Eskimos. The paralytic attack rate among chimpanzees fed virulent strains of poliovirus can be 20 per cent, and among cynomolgus monkeys the feeding of large doses of virulent virus has produced a paralytic rate as high as 75 per cent. How can we by experimental studies establish the probability that a selected strain will not be paralytogenic for human beings even on rare occasions? There were at least three approaches to this question: 1) it was necessary to establish the relative susceptibility

TABLE I.—COMPARATIVE PARALYTOGENIC ACTIVITY (VIRULENCE) IN *INTRACEREBRALLY* INOCULATED CYNOMOLGUS MONKEYS OF TYPE 1 POLIOMYELITIS VIRUS (MAHONEY STRAIN) PROPAGATED IN DIFFERENT WAYS IN CYNOMOLGUS KIDNEY TISSUE CULTURES

Kidney Passage 10 — *Serial Passages with Minimal Inocula*		*Kidney Passage 33* — *Rapid Passages with Large Inocula Followed by Purification by Terminal Dilution Technique*		
No. of TCD_{50} Inoculated*	*No. of Monkeys Paralyzed*	*No. of TCD_{50}* Inoculated*	*No. Showing CNS Lesions*	*No. of Monkeys Paralyzed*
		16,000,000	0/4	0/4
		1,600,000	0/4	0/4
500,000	5/5	160,000	0/4	0/4
50,000	5/5	16,000	0/4	0/4
5,000	5/5	1,600	0/4	0/4
500	5/5	160	0/4	0/4
50	3/5	16	0/4	0/4
5	4/5			
0.5	1/5			

* TCD_{50} = 50 per cent tissue culture cytopathogenic dose

of the nervous system of different primates; 2) it was necessary to find the most highly attenuated strains for the most susceptible primate neurons; and 3) it was necessary to show that strains of the maximum attainable attenuation do not occur in the nervous system of patients with paralysis.

The first important fact we learned was that neurotropism as well as other properties of the polioviruses were not "all or none" characters which were either present or absent in a given strain, but rather that different strains exhibited spectra of activity ranging from high to low which could be measured quantitatively by reference to some fixed property, such as the cytopathogenic effect in monkey kidney cultures. The next important fact to emerge was that the index of neurotropism for primates was a function not only of the virus but also of the neurons among which the inoculum was placed. Thus, the brainstem neurons of the monkey reached by an intracerebral inoculation in the region of the thalamus proved to be more resistant than the lower motor neurons

TABLE II.—SOME PATTERNS FOUND ON INITIAL *INTRACEREBRAL* SCREENING TEST IN MONKEYS OF POLIO STRAINS DERIVED FROM STOOLS OF HEALTHY CHILDREN

Relative Activity in Brainstem Neurons	*Paralysis with Indicated Inocula*	
	10^6 - $10^{7.7}$ TCD_{50}	10^3 - $10^{4.7}$ TCD_{50}
High	3 / 3	3 / 3
Moderate or Low	3 / 3	0 / 3
Mixed Population	1 / 3	2 / 3
Dominantly Inactive (No Lesions) or Very Low — Lesions Present	0 / 3	0 / 3

TABLE III.—SPINAL TESTS IN MONKEYS AND CHIMPANZEES ON POLIO STRAINS THAT ARE INTRACEREBRALLY AVIRULENT FOR MONKEYS

Relative Spinal Activity in Cynomolgus	*Paralytogenic Effect at Indicated Dosages in*	
	Cynomolgus Monkeys	*Chimpanzees*
High	10^2 - 10^3 TCD_{50} ..... most	$10^{6.5}$ to $10^{7.7}$ TCD_{50}
	10^4 TCD_{50} or more ..... almost all	None
Intermediate	10^2 - 10^3 TCD_{50} ..... none	
	10^4 - 10^5 TCD_{50} ..... irregular	None
	10^6 TCD_{50} or more ..... almost all	
Least	10^2 - 10^3 TCD_{50} ..... none	
	10^4 - 10^5 TCD_{50} ..... none	None
	10^6 TCD_{50} or more ..... occasional	

reached directly by an intraspinal inoculation in the lumbar region (Fig. 1), and the lower motor neurons of chimpanzees turned out to be more resistant than those of cynomolgus monkeys. The spectrum of neurotropic activity of different naturally occurring and experimentally segregated strains was found to range from the highest in which one to ten tissue culture doses produce paralysis in intracerebrally inoculated monkeys to the lowest in which even one million tissue culture doses

fail to produce paralysis in large numbers of spinally inoculated monkeys. All grades were found in between (Tables I, II, and III). For example, strains which may be regarded as being attenuated a million times because one million to ten million tissue culture doses fail to produce paralysis or lesions after intracerebral inoculation (Table I), can still be paralytogenic for monkeys when very small doses are injected intraspinally. When these more sensitive lower motor neurons were used as indicators of still greater attenuation a further graded series was demonstrated (Table III). A total of 53 chimpanzees have now been tested by spinal inoculation of large doses of various attenuated strains and not one of them developed paralysis. Some strains which were highly paralytogenic even in small doses on spinal inoculation in monkeys produced neither lesions nor paralysis when maximum doses were injected intraspinally in chimpanzees. On the other hand, of six chimpanzees inoculated intraspinally with large doses of two strains derived from the spinal cord of fatal human cases, five developed varying degrees of paralysis and all exhibited extensive lesions. It is also noteworthy that no virus that is intracerebrally avirulent for monkeys has been found either in the nervous tissue of fatal human cases or in the stools of paralytic cases. In the stools one may occasionally encounter the pattern of mixed populations since a certain proportion of less neurotropic virus particles may arise during the course of propagation in the non-nervous tissue of the alimentary tract just as they have been shown to appear on passage in monkey kidney cultures *in vitro*.

Polioviruses like all other living agents produce a certain number of mutants with different characteristics. If there were no mutants we would have no attenuated viruses with which to work. It is easy to select the more neurotropic mutants by the simple procedure of inoculating millions of infective particles intracerebrally in large numbers of monkeys. Such studies incidentally have shown that mutation in the direction of greater neurotropism occurs in small steps along the spectrum. The problem of selecting for the less neurotropic virus particles has been a very difficult one, since the highly neurotropic polioviruses multiply very well in various kinds of non-nervous tissue of primates or in the nervous system of certain rodents. It still is not clear why serial passages in certain tissue cultures *in vitro* or in certain rodents *in vivo* have yielded populations greatly enriched in attenuated virus particles, unless it be that the mutation pressure in the direction of lesser neuro-

tropism is greater. Separation of the attenuated mutants from the mixed populations was achieved either by the terminal dilution technique in which one or more virus particles are trapped in a single tissue culture tube or in a single animal when highly diluted material is titrated, or optimally by the recently developed plaque technique of Dulbecco and Vogt[4] which permits the isolation of progeny from single virus particles.

Already at the end of 1953 we had strains of each of the three immunologic types of poliovirus which were a million or more times attenuated for the monkey nervous system and nonparalytogenic on spinal inoculation of maximum doses in chimpanzees. The work of the subsequent years was devoted to the testing of a variety of methods of propagation as a means of selecting the most highly attenuated viruses for the most sensitive lower motor neurons of cynomolgus monkeys. Further continuation of rapid passages in cultures of non-nervous tissues of the attenuated strains already segregated by the terminal dilution technique not only failed to yield the desired variants but in one instance yielded virus of even greater neurotropism.[3] The circumstances which might permit such a change became apparent only recently when it was found that in a slightly acid culture medium the more neurotropic virus particles multiplied more extensively than the highly attenuated ones. An extensive search in nature during epidemiologically quiescent periods by obtaining polioviruses from healthy children who had no contact with recognized cases of poliomyelitis yielded a large number of strains ranging from high to relatively low neurotropic activity, but with the exception of one type 2 strain ("P 712" from a healthy child in Louisiana) none was found to be as highly attenuated as the best of those segregated by laboratory manipulations. Forty-nine naturally occurring strains from healthy children were tested in my laboratory, and 20 or more have been tested by Paul, Melnick and Horstmann.[5] Chanock and I tested 18 naturally occurring and laboratory developed attenuated strains of all three types (nine type 1, four type 2, and five type 3) in chick embryo tissue cultures but found none that would multiply. The one type 2 strain that had been adapted to chick embryos by Cox and his associates[6] has retained sufficient neurotropism after 71 passages in chick embryos to produce lesions in most monkeys inoculated intracerebrally with approximately 100,000 mouse infective doses,[7] indicating that the chick embryo does not select against neurotropic poliovirus. After Li and Schaffer[8] picked up the most highly attenuated type 1

TABLE IV.—INVERSE POSITION OF PRIMATES WITH REGARD TO SUSCEPTIBILITY OF NERVOUS SYSTEM AND ALIMENTARY TRACT

Cells	*Most Susceptible* ⟶		*Most Resistant*
Neurons	Monkey (Lower motor),	Monkey (Brainstem),	Chimpanzee (Lower motor), [Man]
Alimentary Tract	Man,	Chimpanzee, Cynomolgus,	Rhesus

virus (the L Sc strain) during the course of alternate passages of an attenuated Mahoney strain in monkey kidney cultures *in vitro* and in the skin of monkeys *in vivo*, Chanock and I studied the behavior of eight attenuated strains of all three types and found that only certain type 2 strains multiplied in the skin of living monkeys [even the type 1 L Sc strain failed to multiply] and that repeated passages through the skin had no significant effect on the neurotropic index.

Before proceeding with an analysis of the neurotropism of a large number of individual virus particles obtained by the plaque technique from the optimum attenuated strain of each type, I should like to summarize some of the studies of the other properties of the various attenuated strains in chimpanzees and human beings which led to their selection for more detailed study. It is obvious that in order to be effective as an immunizing agent by the oral route, a strain must be able to multiply somewhere in the alimentary tract and this property of various polioviruses had to be investigated as quantitatively and intensively as the property of neurotropism. The most important finding to emerge from this study was that the primates occupy an inverse position as regards the susceptibility of the alimentary tract and the nervous system (Table IV), the human intestinal tract being capable of infection by doses of virus which are ineffective in chimpanzees, and chimpanzees being infected by doses which are ineffective in cynomolgus monkeys. Virus taken by mouth does not multiply in the buccal mucosa, gums or tongue but does multiply in the throat and intestinal tract.[3d] Only when the amount of virus that is swallowed is large enough (one million or more tissue culture infective doses) for some of it to lodge in the posterior pharyngeal wall is there regular multiplication in the throat as well as in the intestines. When the dose is smaller (100,000 infective

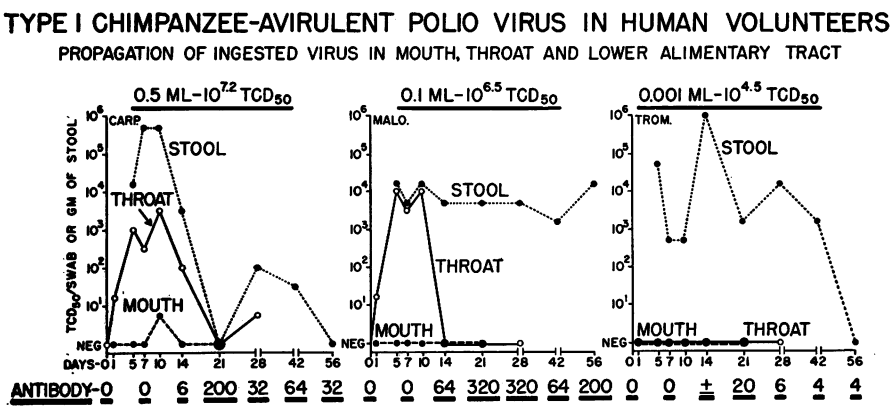

Fig. 2

doses or less) the throat is only irregularly infected, and virus is excreted only in the stools (Fig. 2). Infection limited only to the lower alimentary tract has now been observed in 33 volunteers. Among chimpanzees infected with large doses of attenuated poliovirus there can be extensive multiplication of the virus in the throat without any demonstrable virus in the stools. This is the main reason why ultimate definitive studies on attenuated strains had to be carried out in human beings. Only strains which were attenuated at least one million or more times as measured by the intracerebral test in monkeys and which were nonparalytogenic in maximal doses by the spinal test in chimpanzees were used in human volunteers. In the very first human test it was found that an attenuated type 3 strain which readily multiplied in the intestinal tract failed to multiply or produce an immunogenic effect after intramuscular injection unless the dose was large enough to permit some localization of the absorbed virus in the intestinal tract. Since no evidence was found for the existence of polioviruses that would multiply in the muscle or skin or the regional lymph nodes without also being able to multiply in the intestinal tract, all subsequent human tests were limited to studies on direct infection of the alimentary tract.

Role of Dosage: Our own studies with various strains showed that approximately 10,000 tissue culture infective doses (about 0.001 ml. of culture fluid) would regularly produce an intestinal infection in those who had not the slightest demonstrable amount of homotypic antibody indicative of past infection with the type of virus that was being fed.

Tests with smaller doses of one type 2 strain ("P 712") showed that while some were infected with as little as 100 tissue culture doses others were not infected with 1,000, and because the volunteers used in this test were already immune to types 1 and 3 polioviruses, there is a possibility that immunity to one type may interfere with the multiplication of very small doses of another type. Koprowski[2b] found that as little as two tissue culture doses of his type 1 (SM) strain could initiate an intestinal infection in some but not in others.

Duration of Excretion and Level of Virus Multiplication: The duration of virus excretion has varied greatly in different individuals from a minimum of about ten days to several weeks. The maximum that I observed was 140 days in one volunteer who was fed the partly attenuated Mahoney strain (Mahoney KP 33) and Koprowski[2b] noted excretion for 171 days in a person who was fed his type 1 (SM) strain. There is a suggestion that immunity to type 2 virus may influence the duration of excretion of type 1 virus. Among patients with paralysis peak titers of one million tissue culture doses of virus per gram of stool are not uncommon. I have found similar peak titers in volunteers fed the partly attenuated type 1 Mahoney strain or the naturally occurring attenuated type 1 strains ("P 2149" and "P 2226") and Koprowski[2b] has also reported such large amounts of virus in the stools of certain persons fed his type 1 SM strain. Only the most highly attenuated type 1 L Sc strain has regularly yielded 100 fold lower peak titers in tests on many stool specimens from 25 volunteers who excreted this virus. In almost all instances, in natural, as well as experimental infections, the peak titers occur only during the first seven to ten days.

Interference After Simultaneous Feeding of More Than One Type of Virus: The simultaneous feeding of approximately ten million infective doses of attenuated poliovirus of all three types to chimpanzees resulted in the complete suppression of multiplication and immunogenic effect of only the type 3 virus.[3c] In human beings Koprowski observed that his type 1 virus interfered with the immunogenic effect of the type 2 (TN) strain when the two were administered simultaneously. Using approximately one million tissue culture doses of naturally occurring attenuated strains ("P 2149," "P 712" and "Glenn") I have found no significant interference when mixtures of 1 and 2 (four volunteers), 1 and 3 (three volunteers), 2 and 3 (three volunteers), and in one instance even when all three types were fed simultaneously. However, a delay in

ONE PATTERN OF VIRUS MULTIPLICATION IN HUMAN ALIMENTARY TRACT WHEN 10^5 PLAQUE FORMING UNITS OF 3 IMMUNOLOGIC TYPES OF ATTENUATED POLIOVIRUS WERE FED AT 3-WEEK INTERVALS

Adult Volunteer without Antibody for any of 3 Types of Poliovirus

NUMBERS OVER DOTS INDICATE TYPES OF VIRUS BEING EXCRETED

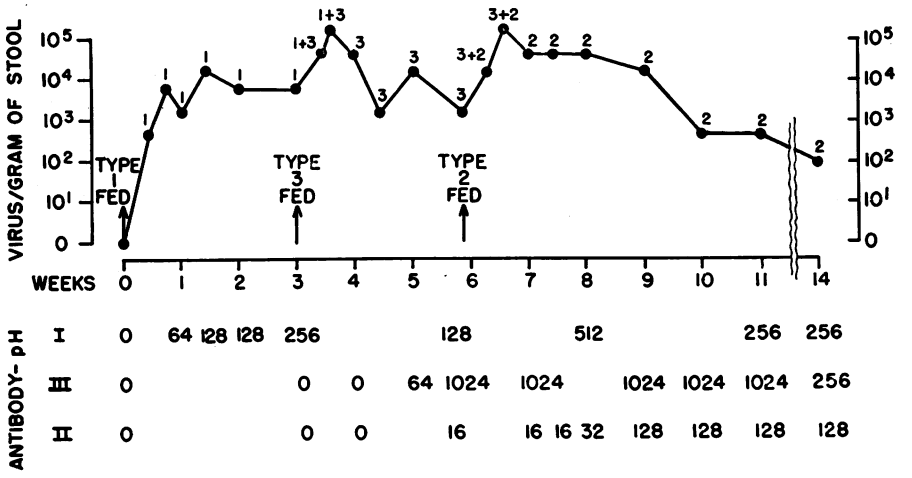

Fig. 3

appearance of antibody and titers in the lower range was observed in some of the men. In the early stages of using a living, attenuated poliovirus vaccine it would be desirable to administer only one type at a time in order that one might have a better chance of identifying a paralytic disease due to spontaneous infection prior to ingestion of the vaccine. Accordingly the feasibility of administering 100,000 infective doses of the optimum available, attenuated strain of each type at three-week intervals was tested in 16 volunteers, and found to be satisfactory. Although some of the volunteers stopped excreting one type of virus before the next type was fed, one pattern obtained in those who were still excreting virus at the time of the next feeding is shown in Figure 3. It can be seen that for a few days, occasionally as long as a week, both types of virus continued to be excreted before the new type became dominant.

Viremia: In five to seven repeated tests for viremia on each of 50 chimpanzees and 72 human volunteers infected with various laboratory-

developed, attenuated strains of all three types, poliovirus was not demonstrated in a single instance during the pre-antibody phase of the infection. With several naturally occurring, attenuated strains transitory traces were occasionally found in the blood of both chimpanzees and human beings. These studies suggest that the viremia observed in nature and in chimpanzees and monkeys experimentally infected with virulent strains, cannot be the result of viral multiplication in the alimentary tract and is probably due to multiplication elsewhere in the body.

Antibody Development: All alimentary infections have been associated with development of antibody, although the actual titers varied with the individual as well as with the strain of virus. During the past year, Chanock and I found that neutralizing antibodies of different avidity appear at different times after natural infection with virulent strains, after experimental infection with attenuated strains and after inoculation with formalinized vaccine. The antibody of low avidity, best demonstrable by the pH test, appears first and its titer can be more than 60 times higher than the antibody of high avidity demonstrated by the cytopathogenic test against 100 infective doses of virus in roller tubes. We have found a certain number of people with natural subclinical infections who have only the low-avidity antibody demonstrable by the pH test and the same was also true of several volunteers infected with the type 1, L Sc strain or the type 3, attenuated Leon virus. Koprowski[2b] reported no significant change in titer of type 2 antibody in a group of 11 individuals four and a half to five and a half years after feeding of his original "TN" strain of reduced intracerebral virulence for monkeys. Our own tests on a group of 11 men one year after feeding one or another of 3 types of attenuated virus showed no significant change in the level of antibody, the low levels remaining low and the high levels remaining high.

Multiplication of Virus in the Alimentary Tract in the Presence of Antibody Acquired in Different Ways: Bodian's experimental studies[9] on monkeys and chimpanzees have shown that the alimentary tract can be infected in the presence of certain levels of passively transmitted antibodies. Koprowski and his associates[2c, 10] showed that the low levels of antibody passively transmitted by small doses of gamma globulin or the higher levels of placentally transmitted antibody in infants did not interfere with the multiplication of attenuated type 1 or type 2 poliovirus in the alimentary tract. Howe's studies[11] on 19 chimpanzees extensively

TABLE V.—EFFECT OF FEEDING 10^5 P.F.U. OF TYPE 1 ATTENUATED POLIOVIRUS (L Sc STRAIN) TO VOLUNTEERS WITH

1) NO HOMOTYPIC ANTIBODY
2) ANTIBODY ACQUIRED FROM 2 DOSES OF SALK VACCINE
3) NATURALLY ACQUIRED ANTIBODY

Group	*No. of Volunteers*	*No. Excreted Virus*	*No. of Days Each Excreted Virus*	*Peak Virus Titers in Stool Log_{10} TCD_{50} Per Gram*
No Antibody	11	11	10, 10, 10, 10, 21+*, 25+ 26+, 26+, 26+, 41, 77	3.7, 3.7, 3.7, 3.7, 3.7, 3.7 4.2, 4.2, 4.2, 4.2, 4.2
Antibody After Salk Vaccine	8	8	9, 10, 10, 13 14, 21+, 26+, 42	2.7, 3.2, 3.4, 3.7 4.2, 4.2, 4.7, 4.7
Naturally Acquired Antibody	8	1	10	4.2

*21+ — Volunteer Fed Another Type of Poliovirus and Excretion of Type 1 was Interfered with.

hyperimmunized with formalinized vaccine indicated that alimentary infection was reduced about 50 per cent after feeding of virulent type 1 virus, but these studies cannot be transposed to humans since in chimpanzees the throat is the dominant site for infection and the throat secretions are known to contain antibody when the serum antibody level is above a certain minimum. My own studies during the past year have shown a marked difference between the resistance of the alimentary tract to infection in those who have acquired their immunity by subclinical infection and those who developed antibody after two doses of Salk vaccine. The data in Table V show that neither the duration of virus excretion nor the peak virus titers per gram of stool were significantly different among the eight volunteers who were fed 100,000 infective doses of type 1 virus after two doses of Salk vaccine (the interval between the two doses was about four weeks and the virus was fed two or three weeks after the second dose) and the 11 volunteers without antibody who were infected with the same dose of the same lot of virus. However, among eight volunteers with varying levels of naturally acquired antibody for all three types, there was no infection

TABLE VI.—LACK OF RELATIONSHIP BETWEEN TITER OF PREEXISTING HOMOTYPIC ANTIBODY AND INTERFERENCE WITH MULTIPLICATION OF TYPE 1 POLIOVIRUS IN HUMAN INTESTINAL TRACT

Antibody After Salk Vaccine (2 Doses)				*Naturally Acquired Antibody*			
	Antibody Titer		*Duration of Excretion Days*		*Antibody Titer*		*Duration of Excretion Days*
Volunteer No.	*High avidity roller tubes*	*Low avidity pH test*		*Volunteer No.*	*High avidity roller tubes*	*Low avidity pH test*	
1	0 ?	16	26+ ?	1	0	4 ?	0
2	±	32	14	2	0	4	0
3	±	128	21+ ?	3	0	8	0
4	3	64	10	4	3	8	10
5	4	256	10	5	4	16	0
6	4+ ?	32	10	6	32	32	0
7	20	256	42	7	200	256	0
8	64	256	14	8	320	512	0

in seven. The data in Table VI show the lack of any relationship between the level of pre-existing antibody, of low or high avidity, and the interference with viral multiplication in the lower alimentary tract. It is possible that the one naturally immune individual in whom the virus multiplied, may not have had a previous type 1 infection—his very low level of type 1 antibody being the result of a group response to his type 2 or 3 infection. Similar results were obtained after feeding of type 2 and type 3 virus after two doses of Salk vaccine, but among the naturally immune controls virus multiplication also occurred in two of six fed the type 2 virus and in three of six who received the type 3 virus. Reinfection tests on volunteers three months after experimental feeding of the type 3, attenuated Leon virus yielded similar results and resistance of the alimentary tract to one million infective doses was observed in a volunteer who had only minimal amounts of low-avidity antibody. These results have a double significance. On the one hand they prove that subclinical infection can occur in those who have had formalinized vaccine, and that it is possible to use a combination of the two types of vaccine, and on the other hand they indicate that a killed virus vaccine alone may be expected to have little effect on the dissemination of polioviruses in nature. The latter conclusion is also supported

by the observations of Lipson, Robbins and Woods[12] and those of Gelfand and his associates[13] on the excretion of poliovirus by Salk-vaccinated children under natural conditions of infection.

Neurotropism of Excreted Virus After Feeding of Various Attenuated Strains: The most important question in these studies is whether attenuated viruses can become sufficiently more neurotropic after propagation in the alimentary tract to constitute a risk not only to the person who ingested the virus but to others to whom it might spread. It would be much easier to obtain the answer if we were dealing with an all or none property rather than with a spectrum of activity which can be measured only by quantitative tests in monkeys. Using extracts of the original stool it is as a rule possible to inoculate only 100 to 10,000 infective doses and a negative result indicates that the predominant viral population in the stool is attenuated to this extent, although here too paralysis of an occasional monkey may only reflect the capacity of the monkey nervous system to select a few more neurotropic particles from the larger inoculum. To permit more definitive tests it has been necessary to increase the amount of virus by growing it in tissue culture before tests are made in monkeys. During the past four years, I have tested 40 chimpanzee and 68 human stools (in a number of instances several specimens from the same individuals), and the cultures derived from them for neurotropism in monkeys. The results were different with different strains depending in part on how extensively attenuated the original virus was and how mixed the population that was fed. The results of these tests as well as those reported by Koprowski[2] in the tests he has carried out with his strains permit only the broad conclusion that reversion to high virulence has not been demonstrated and that the alimentary tract does not selectively favor more neurotropic poliovirus. Since the intestinal tract provides a large surface for viral propagation which can continue for many generations over a period of weeks it is clear that the more mixed the original population and the more neurotropic the bulk of the viral population the more frequently one may expect to encounter virus of greater neurotropism in the stools. This in fact occurred with the least attenuated Mahoney strain as well as with all of the naturally occurring type 1 attenuated viruses. The indication that the alimentary tract does not selectively favor the more neurotropic particles was derived from observations that greater neurotropism of virus in the stools was a chance finding encountered in some and not

in others, sometimes during the first two weeks and not later in the same individual and sometimes the reverse, i.e., not in the early specimens but in the later ones. In the one volunteer who excreted virus for 140 days after ingestion of the partly attenuated Mahoney strain, neither the original stool nor the culture fluid derived from it showed evidence of greater neurotropism at 140 days although the nine day stool specimen did. More highly neurotropic stool cultures which paralyzed monkeys after intracerebral inoculation of 10,000 infective doses were in three instances inoculated intraspinally in doses exceeding one million infective virus particles in 9 chimpanzees without production of paralysis. Nevertheless, in selecting attenuated strains for further study all those which even occasionally yielded stools or stool cultures that produced paralysis in intracerebrally inoculated monkeys at the 10,000 to 100,000 level were eliminated as being either too mixed or too neurotropic. Six of eight type 1 strains that I had tested in chimpanzees or in human volunteers or both (Table VII) and Koprowski's type 1 "SM" strain[2] had to be eliminated on this basis, and only the most highly attenuated L Sc strain originally developed by Li and Schaeffer[8] and further purified in my laboratory proved to be satisfactory. The most suitable candidate for the type 2 strain was segregated from the stool of a healthy child sent to me by Doctors Fox and Gelfand of New Orleans and designated as "P 712," and the type 3 strain which fulfilled these requirements was an attenuated derivative of the original Leon virus segregated in my laboratory.[3] The type 2 strains of Koprowski and his associates[2c, 7] were eliminated chiefly because of their greater neurotropism.

Stability on Propagation in Large Lots: The stability of these three selected strains on large scale cultivation in monkey kidney was tested by preparing 20 liter lots of each. Various tests failed to reveal any simian cytopathogenic agents in these 60 liters of culture fluid. The intracerebral and intraspinal titrations in monkeys as well as the spinal tests in a total of 15 chimpanzees yielded results that were identical with those previously obtained with the seed lots. These lots were employed in 53 human volunteers, establishing among other things the effectiveness of 0.01 ml. of culture fluid as a standard dose. Despite this demonstrated stability on large scale cultivation an analysis of a large number of individual virus particles, in the form of progeny from triply-purified plaques, showed that the original seed viruses were not homogeneous,

TABLE VII.—SCREENING OF 13 ATTENUATED STRAINS OF POLIOVIRUS BY TESTS IN ALIMENTARY TRACT OF CHIMPANZEES AND MEN

Type	*Strain*	*Spinal Activity in Monkeys*	*No. Tested*		*Results of Alimentary Infection*				*Preference for Oral Vaccine*
			Chimp.	*Men*	*Antibody*	*Viremia*	*Alimentary Multiplic.*	*Stability of Virus*	
I	Brunhilde-Enders	High	4	—	+++	0	+++	Yes	No
	Mahoney-LSa-mouse-Cinci	Low	3	—	0 to +	0	+	Yes	No
	" -KP 33	High	8	10	+++	0	+++	No	No
	" -L Sc-Cinci	Lowest	3	33	++	0	++	Yes	Yes!
	Cleveland "80-4" *	Intermed.	3	—	+++	Slight	+++	No	No
	New Orleans "P 1553" *	"	3	—	+++	"	+++	No	No
	" " "P 2226" *	"	—	6	++++	"	+++	No	No
	" " "P 2149" *	Low	—	14	++++	"	+++	No	No
II	Y-SK—KP 51	Intermed.	14	14	+++	0	+++	No	No
	Cincinnati—"FAF 117" *	High	3	—	+++	Slight	+++	No	No
	New Orleans—"P 712" *	Low	—	38	+++	trace-rare	+++	Yes	Yes!
III	Leon—KP 34 and 37	Low	8	44	++	0	+++	Yes	Yes!
	Cincinnati—"Glenn" *	High	3	13	++	0	+++	Yes	No

* Naturally occurring strains. Underlined properties eliminated strain.

TABLE VIII.—TYPE 3 POLIOVIRUS—ISOLATION OF PARTICLES WITH VARYING NEUROTROPIC ACTIVITY FROM HIGHLY ATTENUATED VIRUS POPULATION PREVIOUSLY PURIFIED BY TERMINAL DILUTION TECHNIQUE

Material Tested		*Paralysis in Cynomolgus Monkeys Inoculated Intraspinally with Approximately Indicated No. of Tissue Culture Infective Doses*		
		1,000,000	100,000	10,000
Leon, KP 34		3/4	1/4	1/4
Leon, KP 36 *		6/9	5/17	0/17
Progeny of Single Particles from Leon, KP 34	Plaque 1 *	0/29	0/14	0/14
	" 2 *	0/15	0/15	0/10
	Plaques 3, 4, 5, 6	3/5 or 4/5	2/5 or 3/5	
	Plaques 7, 8, 9	4/5 or 5/5	5/5 or 4/5	

* Composite of tests on several different lots.

and that still more highly attenuated viruses could be isolated.

Neurotropic Activity of Progeny of Individual Virus Particles Recovered from Optimum Attenuated Strains: The data summarized in Table VIII show the results obtained with the type 3 strain. Reproducible spinal titrations in monkeys were obtained with cultures grown from the original seed virus in large or small amounts with virus inocula of different size, but the progeny obtained from nine individual particles yielded entirely different results. Three (plaques 7, 8 and 9) were distinctly more neurotropic, four (plaques 3, 4, 5 and 6) were similar in activity to the parent population, and two of the plaques yielded for the first time virus which in the largest doses was not paralytogenic in large numbers of monkeys. The results obtained with the type 2 virus (Table IX) showed a similar inhomogeneity and the progeny from one of the nine plaques tested (plaque 1) was distinctly superior to all the others.

TABLE IX.—TYPE 2 POLIOVIRUS—ISOLATION OF PARTICLES WITH VARYING NEUROTROPIC ACTIVITY FROM HIGHLY ATTENUATED VIRUS POPULATION PREVIOUSLY PURIFIED BY TERMINAL DILUTION TECHNIQUE

Material Tested		*Paralysis in Cynomolgus Monkeys Inoculated Intraspinally with Approximately Indicated No. of Tissue Culture Infective Doses*		
		1,000,000	100,000	10,000
"P 712", KP_4		2/4	0/8	0/4
"P 712", KP_6*		6/17	5/17	4/17
Progeny of Single Particles from "P 712", KP_4	Plaque 1 *	2/24 slight	0/19	1/19 slight
	Plaque 2	5/10	2/5	4/5
	Plaques 3 to 9	3, 4 or 5/5	2, 3 or 4/5	

* Composite of tests on several different lots.

Subsequent passages in cultures at a pH of 7.6 to 7.8 yielded virus that was nonparalytogenic in the largest doses. It should be pointed out here that these nonparalytogenic variants still have a certain minimal residual neurotropism, for while some of the monkeys show no specific lesions of any kind even in the gray matter adjacent to the inoculum, focal polio lesions are present in others. These new single particle strains have been fed to ten chimpanzees and produced immunogenic alimentary infections in all.

The type 1, L Sc strain which already possessed such low activity that monkeys inoculated intraspinally with one million infective doses only irregularly exhibited slight localized, often only transitory, paralysis also revealed somewhat different patterns among the progeny derived from ten different plaques (Table X). Particularly noteworthy was the fact that the progeny of some of the virus particles (especially plaques 3 and 4) exhibited the zone phenomenon, while others did not. The zone phenomenon,[3b] is characterized by the appearance of paralysis in monkeys inoculated with smaller doses and not with the larger ones. The progeny of plaque 1, devoid of this zone phenomenon, was dis-

TABLE X.—TYPE 1 POLIOVIRUS—ISOLATION OF PARTICLES WITH VARYING NEUROTROPIC ACTIVITY FROM HIGHLY ATTENUATED VIRUS POPULATION PREVIOUSLY PURIFIED BY TERMINAL DILUTION TECHNIQUE

Material Tested		*Paralysis in Cynomolgus Monkeys Inoculated Intraspinally with Approximately Indicated No. of Tissue Culture Infective Doses*		
		1,000,000	100,000	10,000
L Sc, Cinci KP_1 to KP_5		4/20	0/8	0/10
L Sc, Cinci KP_7		5/17 slight	2/17	1/17
Progeny of Single Particles from L Sc, KP_5	Plaque 1	2/10 transitory	0/10	0/5
	Plaque 2	1/10 transitory	2/10	1/5 transitory
	Plaque 3	1/10 slight	0/5	2/5
	Plaque 4	0/10	2/5	3/5
	Plaques 5, 6, 7	1/5	2/5	
	Plaques 8 and 9	2 or 3/5	1 or 2/5	
	Plaque 10	3/5	3/5	

tinctly superior to all the others. Similar tests in monkeys with the progeny of 14 plaques from the two optimum naturally occurring, attenuated type 1 strains ("P 2149" and "P 2226") showed that all of them were highly paralytogenic intraspinally both at the one million and 100,000 dose levels. Still another attempt to obtain type 1 virus that might be superior to that derived from the best L Sc plaque was made by an analysis of four individual plaques derived from a human stool obtained 15 days after ingestion of the original L Sc seed virus. This stool culture had the same low activity intraspinally in monkeys as the progeny of the best L Sc plaque and was also devoid of a zone phenomenon. The viruses recovered from these 4 plaques were similar in their activity to that of the parent culture but not superior to the best L Sc plaque.

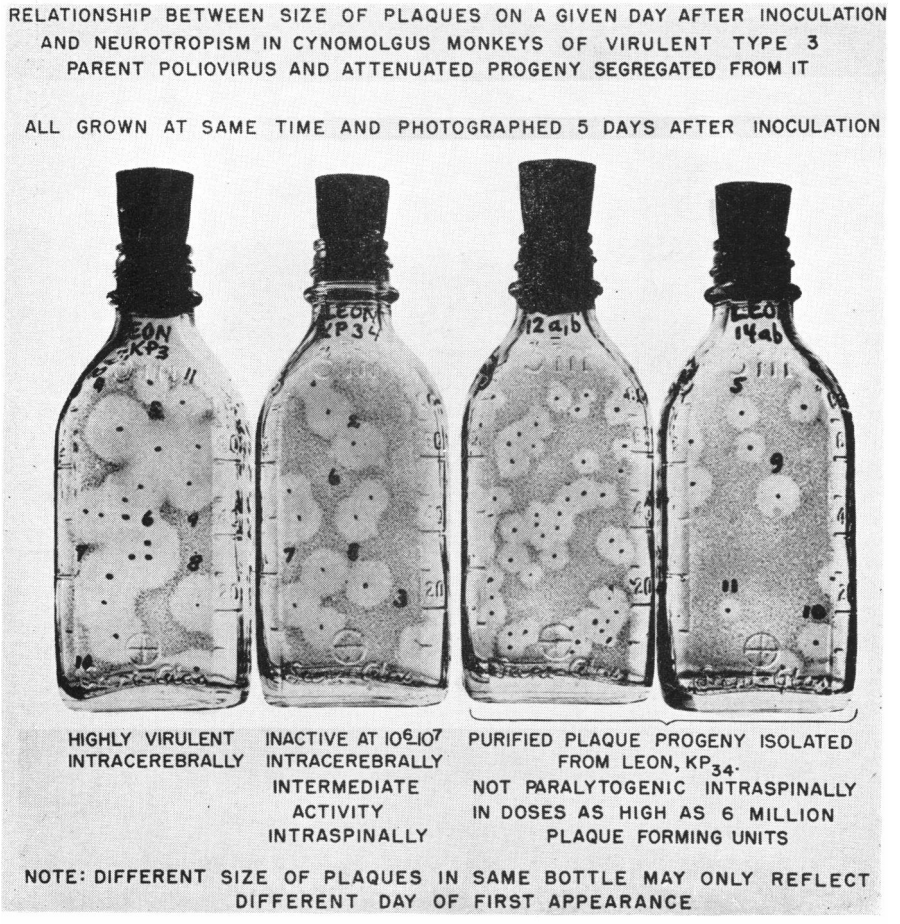

Fig. 4

Relationship between Plaque Size and Neurotropism: A definite relationship between neurotropism and plaque size *in vitro* has been found for the type 3 and 2 but not for the type 1 viruses. Under the special conditions in the rubber-stoppered plaque bottles prepared by the technique of Hsiung and Melnick[14] the smallest plaques were produced by the most highly attenuated strains. Figure 4 shows the sizes of plaques at the same time after inoculation of the original highly virulent type 3 Leon virus, of the attenuated derivative segregated from it after many passages in monkey cultures, and of the nonparalytogenic progeny of the two particles that were in turn segregated from it.

TABLE XI.—INFLUENCE OF pH OF MEDIUM AT TIME OF INOCULATION ON YIELD OF INFECTIVE VIRULENT AND ATTENUATED POLIOVIRUS PARTICLES PER CELL IN MONKEY KIDNEY CULTURES

1 P.F.U. of Virus Added per 100 Epithelial Cells

Type	*Strain*	*Virulence for Monkeys*	*Hours for Necrosis of All Cells*		*Yield of Virus P.F.U./ML*		*Infective Virus Per Cell*	
			pH 6.8	pH 7.8	pH 6.8	pH 7.8	pH 6.8*	pH 7.8**
2	YSK, KP_{10}	High Intracerebral	38	38	2.6×10^7	7×10^7	350	700
	"P 712", 10 ab_1	Lowest Spinal	80	44	3×10^6	2.4×10^7	40	240
3	Leon, KP_3	High Intracerebral	64	48	2.1×10^7	1.2×10^8	280	1200
	Leon, 12 a_1b	None at 10^6 P.F.U. Spinal	96	48	3.2×10^6	3.2×10^7	43	320
	Leon, 14 ab	None at 10^6 P.F.U. Spinal	72	48	5.2×10^6	4.5×10^7	69	450

* 150,000 cells present in control pH 6.8 roller tubes at time of beginning cytopathogenic effect.
** 200,000 cells present in control pH 7.8 roller tubes at time of beginning cytopathogenic effect.

Relationship between pH of Culture Medium and Yield of Virus by Virulent and Attenuated Strains: During the course of our work with various attenuated strains it was observed that the yield of virus was at least tenfold less when they were propagated in tissue cultures that had metabolized sufficiently to reduce the pH of the medium to 6.8 or less. Dulbecco and Vogt,[15] working with strains of known neurotropism from my laboratory, recently discovered a distinct relationship between neurotropism and plating efficiency at low and high concentrations of bicarbonate. They found that while the virulent viruses yielded as many plaques on the "acid" as on the "alkaline" plates, the more attenuated the strains were the fewer plaques they produced on the "acid" plates at a given time after inoculation. These differences (as high as one million fold) were most marked with our newly isolated single plaque strains. Tests which I have carried out (Table XI) showed that while with virulent strains the yield of infective virus per cell was only slightly greater at pH 7.8 (high bicarbonate reserve) than at pH

6.8 (low bicarbonate reserve), it was 6 to 8 fold less at pH 6.8 for the most highly attenuated single plaque strains. This means that propagation at an acid pH would favor the more neurotropic mutants that might emerge, and conversely that propagation at an appropriate alkaline pH would prevent the enrichment of such mutants in the culture medium.

In conclusion it may be said that much has been learned about the basic principles underlying immunization with attenuated polioviruses by the oral route, and that we now have strains derived from single particles of virus that are sufficiently highly attenuated and stable under appropriate conditions of cultivation to justify their use for the next stepwise studies on immunization of human beings. Experience has taught me the importance of accentuating the negative—and I therefore wish to stress that we are not ready for so-called mass trials of an oral vaccine but only for those tests on increasingly larger groups which must precede any consideration of tests on a large scale. I also want to accentuate the positive by saying that the Salk vaccine is the only polio vaccine available for public use at this time and that advantage should be taken of its protective effects to the maximum extent of its availability. I may add that some reservations that I had before disappeared with the demonstration that the antibodies and immunity produced by the Salk vaccine do not interfere with the alimentary infection produced by an oral attenuated vaccine or naturally acquired virus. This then is as far as we have gone, and it is obvious that we still have a great deal to learn before the ultimate goal of complete elimination of poliomyelitis is achieved.

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REFERENCES

1. Koprowski, H., Jervis, G. A. and Norton, T. W. Immune responses in human volunteers upon oral administration of a rodent-adapted strain of poliomyelitis virus, *Amer. J. Hyg. 55*:108-28, 1952.
2. Koprowski, H., Jervis, G. A. and Norton, T. W. Administration of attenuated type 1 poliomyelitis virus to human subjects, *Proc. Soc. exp. Biol. Med. 86*: 244-47, 1954.
a) Koprowski, H. Immunization of man against poliomyelitis with attenuated

preparations of living virus, *Ann. N. Y. Acad. Sci. 61*:1039-49, 1955.

b) Koprowski, H. Living attenuated poliomyelitis virus as an immunizing agent of man, *So. Afr. med. J. 29*: 1134-42, 1955.

c) Koprowski, H. and others. Clinical investigations on attenuated strains of poliomyelitis virus; use as a method of immunization of children with living virus. *J. Amer. med. Assoc. 160*:954-56, 1956.

3. Sabin, A. B., Hennessen, W. A. and Winsser, J. Studies on variants of poliomyelitis virus; experimental segregation and properties of avirulent variants of three immunologic types, *J. exp. Med. 99*:551-76, 1954.

a) Sabin, A. B. Etat actuel des recherches sur la vaccination contre la poliomyélite, *Bruxelles-Méd. 34*:1413-26, 1954. English translation and addendum in *Bol. Ofic. Sanit. Panamericana 37*:711-26, 1954.

b) Sabin, A. B. Characteristics and genetic potentialities of experimentally produced and naturally occurring variants of poliomyelitis virus, *Ann. N.Y. Acad. Sci. 61*:924-39, 1955.

c) Sabin, A. B. Immunization of chimpanzees and human beings with avirulent strains of poliomyelitis virus, *Ann. N.Y. Acad. Sci. 61*:1050-56, 1955.

d) Sabin, A. B. Behavior of chimpanzee-avirulent poliomyelitis viruses in experimentally infected human volunteers, *Brit. med. J. 2*:160-62, 1955; also in *Amer. J. med. Sci. 230*:1-8, 1955.

e) Sabin, A. B. Pathogenesis of poliomyelitis—reappraisal in the light of new data, *Science 123*:1151-57, 1956.

4. Dulbecco, R. and Vogt, M. Plaque formation and isolation of pure lines with poliomyelitis viruses, *J. exp. Med. 99*: 167-82, 1954.

5. Paul, J. R., Melnick, J. L. and Horstmann, D. Personal communication.

6. Roca-Garcia, M., Moyer, A. W. and Cox, H. R. Poliomyelitis II. Propagation of MEF_1 strain of poliomyelitis virus in developing chick embryo by yolk sac inoculation, *Proc. Soc. exp. Biol. Med. 81*:519-25, 1952.

7. Roca-Garcia, M., Koprowski, H., Jervis, G. A., Norton, T. W., Nelson, T. L. and Cox, H. R. Immunization of humans with chick embryo adapted strain of MEF_1 poliomyelitis virus, *J. Immunol. 77*:123-31, 1956.

8. Li, C. P. and Schaffer, M. Isolation of a non-neurotropic variant of type 1 poliomyelitis virus, *Proc. Soc. exp. Biol. Med 87*:148-53, 1954.

9. Bodian, D. Experimental studies on passive immunization against poliomyelitis, passive-active immunization and pathogenesis after virus feeding in chimpanzees, *Amer. J. Hyg. 58*:81-100, 1953.

10. Koprowski, H. and others. Immunization of infants with living attenuated poliomyelitis virus, *J. Amer. med. Assoc. 162*:1281-88, 1956.

11. Howe, H. A. Poliomyelitis infection in immunized chimpanzees. *Ann. N. Y. Acad. Sci. 61*:1014-22, 1955.

12. Lipson, M. J., Robbins, F. C. and Woods, W. A. The influence of vaccination upon intestinal infection of family contacts of poliomyelitis patients (abstract), *J. clin. Invest. 35*:722, 1956.

13. Gelfand, H. M., LeBlanc, D. R. and Fox, J. P. Observations on natural poliovirus infections in children, *Amer. J. pub. Health*, in press.

14. Hsiung, D. G. and Melnick, J. L. Plaque formation with poliomyelitis, Coxsackie, and orphon (Echo) viruses in bottle cultures of monkey epithelial cells. *Virology. 1*:533-35, 1955.

15. Dulbecco, R., Vogt, M. and Sabin, A. B., unpublished studies.